Gau 1803
1640

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, United States Patent and Trademark Office, Washington, DC. 20231, on this 23 day of July, 1998.

Marianne H. Michel

CASE NO. 0448

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Zhao, Zuo-Yu; Gu, Weining; Cai, Tishu; and Pierce, Dorothy A.

Serial No. 08/963,096 Group Art Unit: 1803

Filed: November 3, 1997 Examiner: Benzion, G.

For: "METHODS FOR AGROBACTERIUM-MEDIATED TRANSFORMATION"

Assistant Commissioner for Patents
Washington, D.C. 20231

DECLARATION OF ZUO-YU ZHAO UNDER 37 CFR § 1.132

To the Assistant Commissioner for Patents
Washington, DC. 20231

Dear Sir:

Zuo-Yu Zhao hereby declares and says:

1. I am a citizen of China and a permanent resident of The United States of America, and I reside in Johnston, Iowa.
2. I am a co-inventor of the above-identified application.
3. I am a permanent, full-time employee of the assignee of the above-identified patent application, Pioneer Hi-Bred International, Inc. Since Nov. 6, 1990 I have held the position of Research Manager.

4. I have a B.S. in Agricultur Sciences, grant d in 1969 by Beijing Agriculture University, Beijing, China.
5. I have a Ph.D. in Biology, granted on Dec. 20, 1987, by Illinois State University.
6. I have eight years' experience in Transformation at the commercial research level.
7. I was a post-doc. at the Cold Spring Harbor Laboratories from Feb. 1988 to Oct. 1990.
8. I am personally familiar with the subject matter of this application and I am making this Declaration to support the claims in this application.
9. I am personally familiar with the method of collection of the data employed in the exhibits.
10. Data in the exhibits were obtained using methods according to the application.
11. The line PHP38 was inadvertently identified as PHP28 in the corresponding application. This typographical mistake is corrected in an amendment in the accompanying office action response.
12. Maize was transformed with both Gus and bar genes in the plasmid using *Agrobacterium*-mediated methods disclosed in the above-identified application. The transgene was shown in Figure 1C in the above-identified application.
13. Data was obtained using the transformation methods described in the present application. Hi-II was transformed with the methods described in Examples 4 and 5 (page 31 - 49 of the present application). PHN46, PHJ90 and PHP38 were transformed with the methods described in Example 7 (pp. 42-45).
14. Southern blots were performed by the method described in "Molecular Cloning" Eds. J, Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, 1989. Southern data on Hi-II (Exhibits A & E), PHN46 (Exhibit B), PHJ90 (Exhibit F) and PHP38 (Exhibit G) stable transgenic T0 plants were derived from *Agrobacterium*-mediated immature embryo transformation prepared according to the present claimed invention.
15. Gus staining in xhibit C was performed by th method described by McCabe, D.E., 1988, *Biotechnol.* 6:923-926.
16. Liberty painting in data not shown was done by the methods of described by

W. Gordon-Kamm tc., 1990, The Plant Cell, 603-618.

17. Gene segregation analysis in Exhibit D was done by the methods described by W. Gordon- Kamm etc., 1990, The Plant Cell, 603-618.

18. With regard to transformation frequency, 39.8%, 7.9%, 1.9% and 1.3% of embryos were transformed in Hi-II, PHN46, PHJ90 and PHP38, respectively.

Exhibit A - Exhibit A is a Southern blot of 13 independently derived, *Agrobacterium* transformed,T0 Hi-II plants using bar as the probe. Lane 15 is a non-transformed control, the first and last lanes are molecular markers and the plasmid copy number control is in lanes 16 and 17. The results shown in this exhibit demonstrate that the T-DNA containing the bar gene was stably integrated into the maize genome of Hi-II and that each independent transformation event exhibited a unique T-DNA integration pattern.

Exhibit A is representative of data including Southern blots of 157 T0 plants that represent at least 107 independent stable transformation events in Hi-II.

Exhibit B- Exhibit B is a Southern blot of 14 independently derived, *Agrobacterium* transformed T0 PHN46 plants using Gus as the probe. Lane 16 is a non-transformed control, the first and last lanes are molecular markers and the plasmid copy number control is in lanes 17 and 18. These results demonstrate that the T-DNA containing the Gus gene was also stably integrated into the maize genome of PHN46 and independent transformation events show unique T-DNA integration patterns.

Exhibit B is representative of data including Southern blots of 140 T0 plants that represent at least 90 independent stable transformation events in PHN46.

Exhibit C-Exhibit C demonstrates data of Gus (X-gluc) staining of corn calli and leaves and Bialaphos resistant T0 plants of stably transformed Hi-II. Data include:

1) Gus stain of Hi-II calli (blue colored tissue) either at Selection-1 (about 3 weeks of selection) or at Selection-3 (about 8 weeks of selection). The Gus gene used in the transformation cassette contained a potato intron (Figure 1 in the present application) within Gus open reading frame (called intron/Gus) which allows Gus gene expression in plant tissue only, but not in *Agrobacterium*. Therefore, the Gus expressing calli are a confirmation of stable transformation of maize tissue by *Agrobacterium*.
2) Hi-II T0 plants from test tubes shown in Exhibit C were regenerated and grown with the medium that contained Bialaphos, a plant herbicide, (a plant expressing the bar gene is resistant to this herbicide). This is another confirmation of the stable transformation of the Hi-II T0 plants by *Agrobacterium*. These T0 plants are representative of 107 independent stable events in Hi-II.
3) Gus staining of the leaves from T0 plants are representative of 107 independent stable events in Hi-II. These results demonstrate that the T-DNA Gus gene can be expressed in corn plants. This special Intron/Gus construction precludes Gus gene expression in *Agrobacterium* and allow its expression in plant cells only. These Gus expressing leaves are strong evidence that corn plants were stably transformed with *Agrobacterium*.

Similar data were obtained in PHN46 and PHJ90.

Exhibit D-Exhibit D contains data analyzing the T1 generation of *Agrobacterium* transformed Hi-II. The table in Exhibit D summarizes the results of T0 self and T0 X Hi-II crosses. Segregation is very close to the expected values of 3:1 and 1:1 respectively. Data show Mendelian segregation pattern both bar and Gus in T1.

Gene segregation data in T1 generations not shown includes: 276 T1 plants that represent 6 independent transformation events were analyzed for gene segregation of both bar and Gus. Gus staining and Liberty painting of these plants

confirmed that the transgenes (bar and Gus) were transmitted to the next generation through both male and female gametes.

Exhibit E- Exhibit E shows the Southern blot results of two of the events described in Exhibit D (Gus probe, *Agrobacterium* transformed T0 and T1 plants). Lanes 1 and 18 are molecular size markers, the plasmid copy controls are in lanes 16 and 17, and the non-transformed control in lane 15. Data from the first set of T0 parents and T1 progeny show that both the T0 plant and the T1 progeny have two bands. This infers that there are 2 copies of Gus that hybridize with the Gus probe suggesting that the 2 copies are linked. Data from the second set of T0 parents and T1 progeny show that the two Gus bands of the T0 parent segregate in the T1 progeny. This data suggest that in this event, the 2 bands represent 2 copies of the transgene that are not linked.

Southern data of these 6 independent events confirm transgenic transmission (bar and Gus).

Exhibit F- Exhibit F is a Southern blot of 7 independently derived, *Agrobacterium* transformed T0 PHJ90 plants using Gus as the probe. Lane 16 is a non-transformed control, the first and last lanes are molecular markers and the plasmid copy number control is in lanes 17 and 18. Lane 4-6 and lane 8 are Gus negative controls. T0 plants in lane 11,12 and 15 were from the same embryo and represent the same event, as do lanes 13 and 14. These results demonstrate that the T-DNA containing the Gus gene was stably integrated into the maize genome of PHJ90. Independent transformation events show different T-DNA integration patterns confirming their unique origin.

Exhibit G- Exhibit G is a Southern blot of 11 T0 plants that represent 9 independently derived, *Agrobacterium* transformed T0 PHP38 plants using bar as the probe. Lane 16 is a non-transformed control, the first and last lanes are molecular markers and the plasmid copy number control is in lanes 17 and 18. Lane 7, 12 and 14 are negative

control, Liberty sensitive plants (no bar gene). Lanes 2-4 are the T0 plants deriv d from the same embryo, representing the same ev nt. These results demonstrate that the T-DNA containing the bar gene was also stably integrated into the maize genome of PHP38 and that independent transformation events show unique T-DNA integration patterns.

19. Data, which is not shown in the present exhibits, were obtained for Liberty (herbicide) painting of corn leaves of

Agrobacterium transformed T0 plants. These results demonstrate that the bar gene that was from T-DNA can be expressed in corn plants. These bar expressing plants are strong confirmation that corn was transformed stably with *Agrobacterium*. Data include:

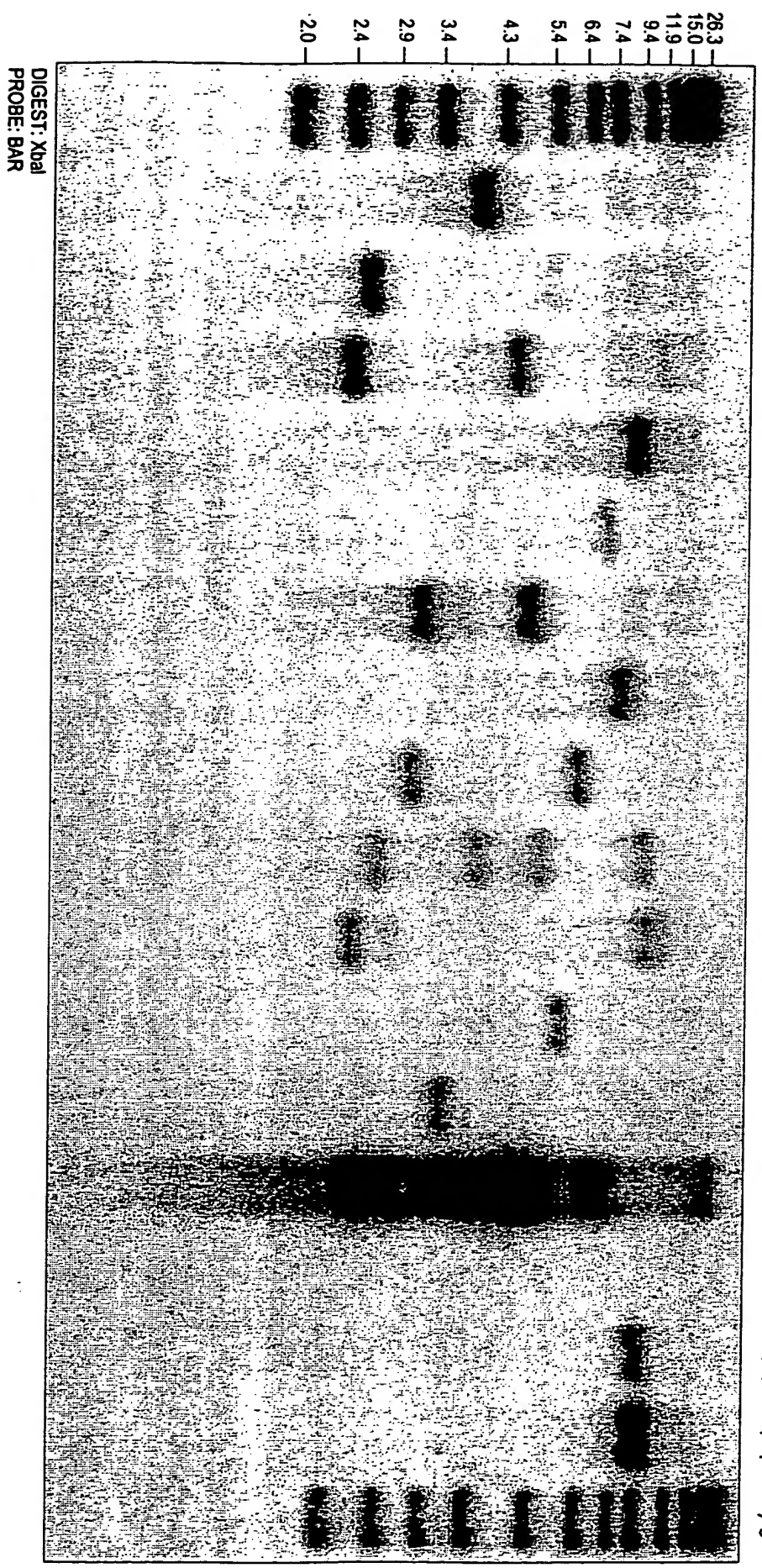
1) Liberty painting of T0 plants that represent 107 independent stable events in Hi-II.
2) Liberty painting of T0 plants that represent 95 independent transformation events in PHN46.
3) Liberty painting of T0 plants that represent 7 independent transformation events in PHJ90.
4) Liberty painting of T0 plants that represent 9 independent transformation events in PHP38.

I further declar that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that all statements made herein were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing hereon.

Zuo-Yu Zhao

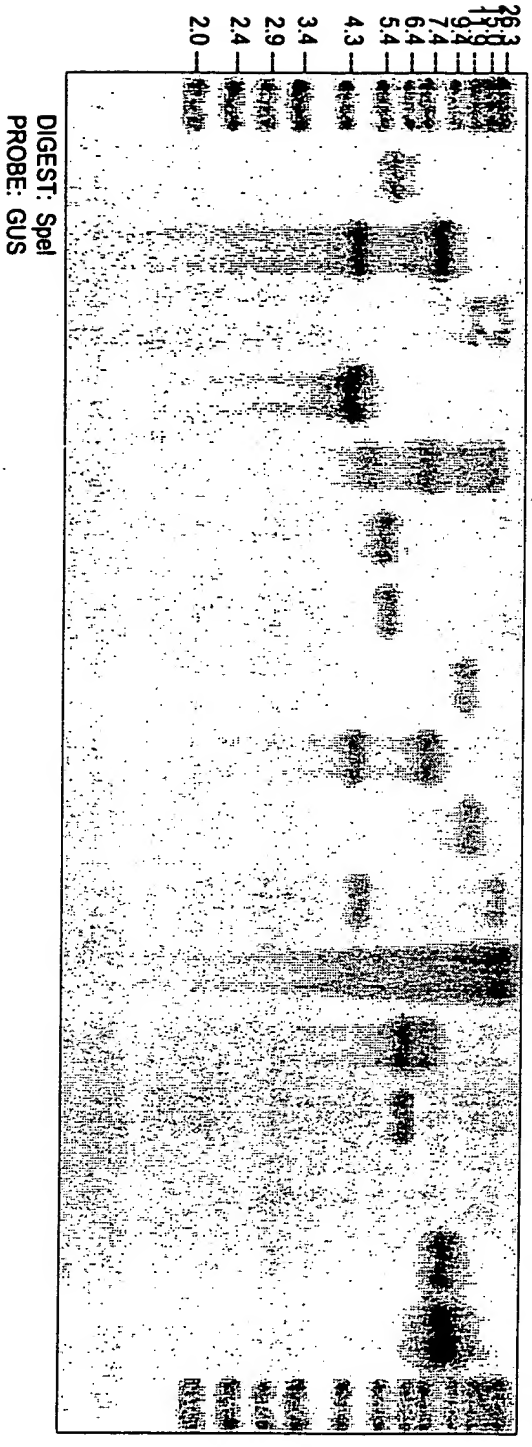
Dated: June 30, 1998

EXHIBIT A



Hi-II

EXHIBIT B



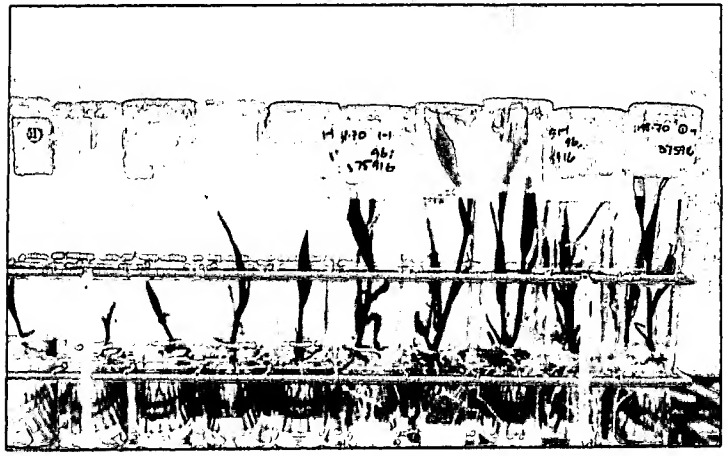
PHN46

EXHIBIT C

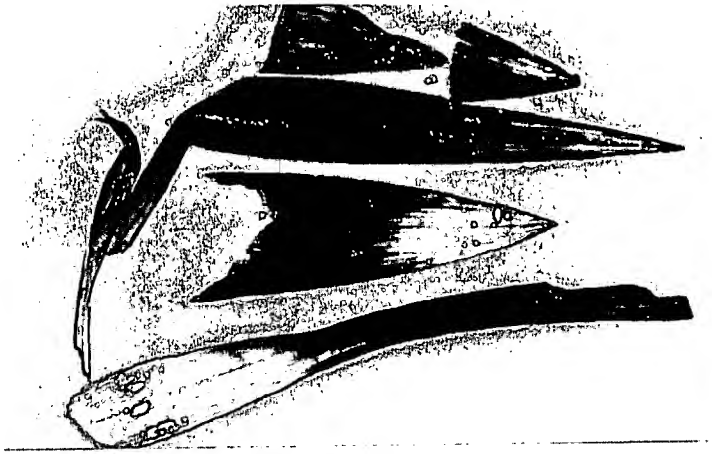
Stable Transformants in HiII

GUS+ sectors at Selection-1

GUS+ Calli at Selection-3



T0 plants generated under Bialaphos selection



GUS expressed in the leaves of these T0 plants

EXHIBIT D

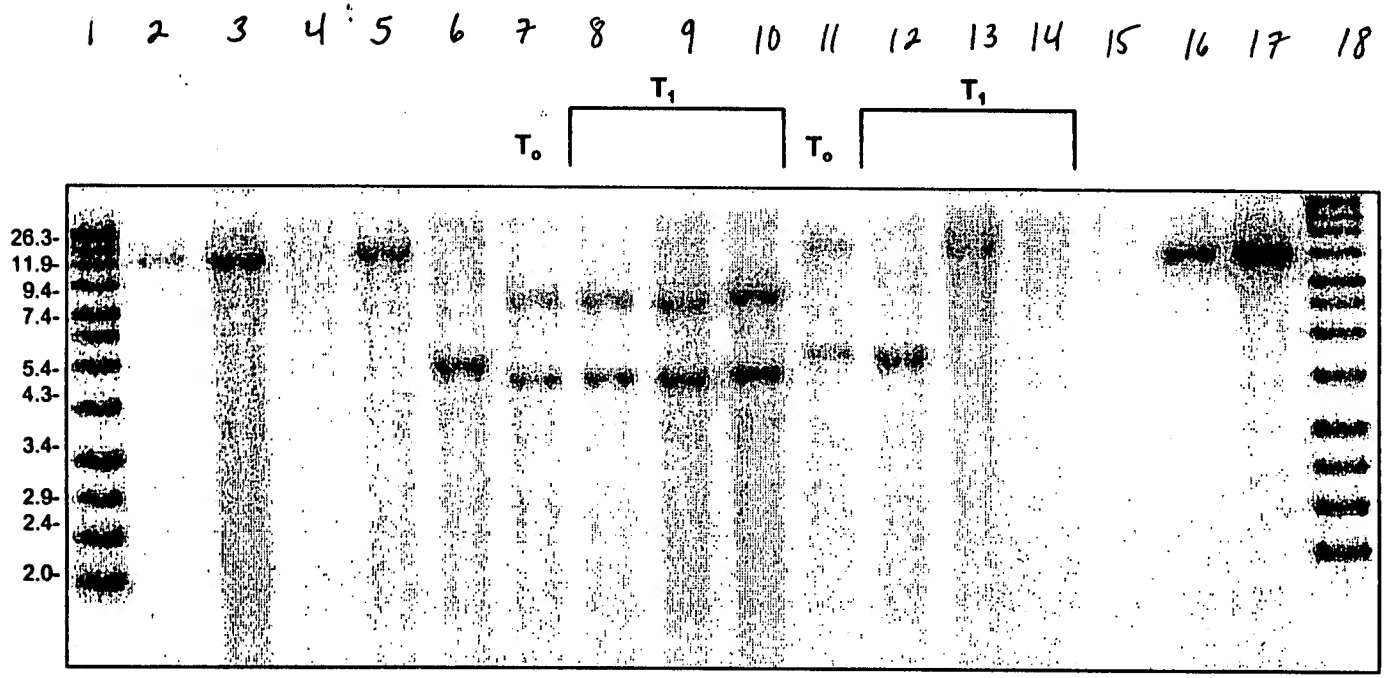
Gene segregation data of T1 plants derived from the Agrobacterium-mediated transformed T0-plants

Summary f the bar and Gus gene segregation in T1 plants

Event No	T0 PLANT No	Cross	No. of T1 Plants	Liberty Painting (bar gene)	X-gluc stain (Gus gene)	Expected
1	1	T0 self	20 plants	17(+) : 3(-)	17(+) : 3(-)	
	2	T0xHi-II	25 plants	6(+) : 19(-)	6(+) : 19(-)	
2	1	T0 self	26 plants	15(+) : 11(-)	15(+) : 11(-)	
	2	T0xHi-II	27 plants	14(+) : 13(-)	13(+) : 14(-)	
3	1	T0 self	31 plants	21(+) : 10(-)	21(+) : 10(-)	
	2	T0xHi-II	32 plants	27(+) : 5(-)	27(+) : 5(-)	
4	1	T0 self	31 plants	20(+) : 11(-)	20(+) : 11(-)	
	2	T0xHi-II	29 plants	12(+) : 17(-)	12(+) : 17(-)	
5	1	T0 self	31 plants	27(+) : 4(-)	27(+) : 4(-)	
6	1	T0xHi-II	24 plants	19(+) : 5(-)	19(+) : 5(-)	
SUM		**T0 Self**	**139**	**100(+) : 39(-)**	**100(+) : 39(-)**	**3 : 1**
		T0 x Hi-II	**137**	**78(+) : 59(-)**	**77(+) : 60(-)**	**1 : 1**

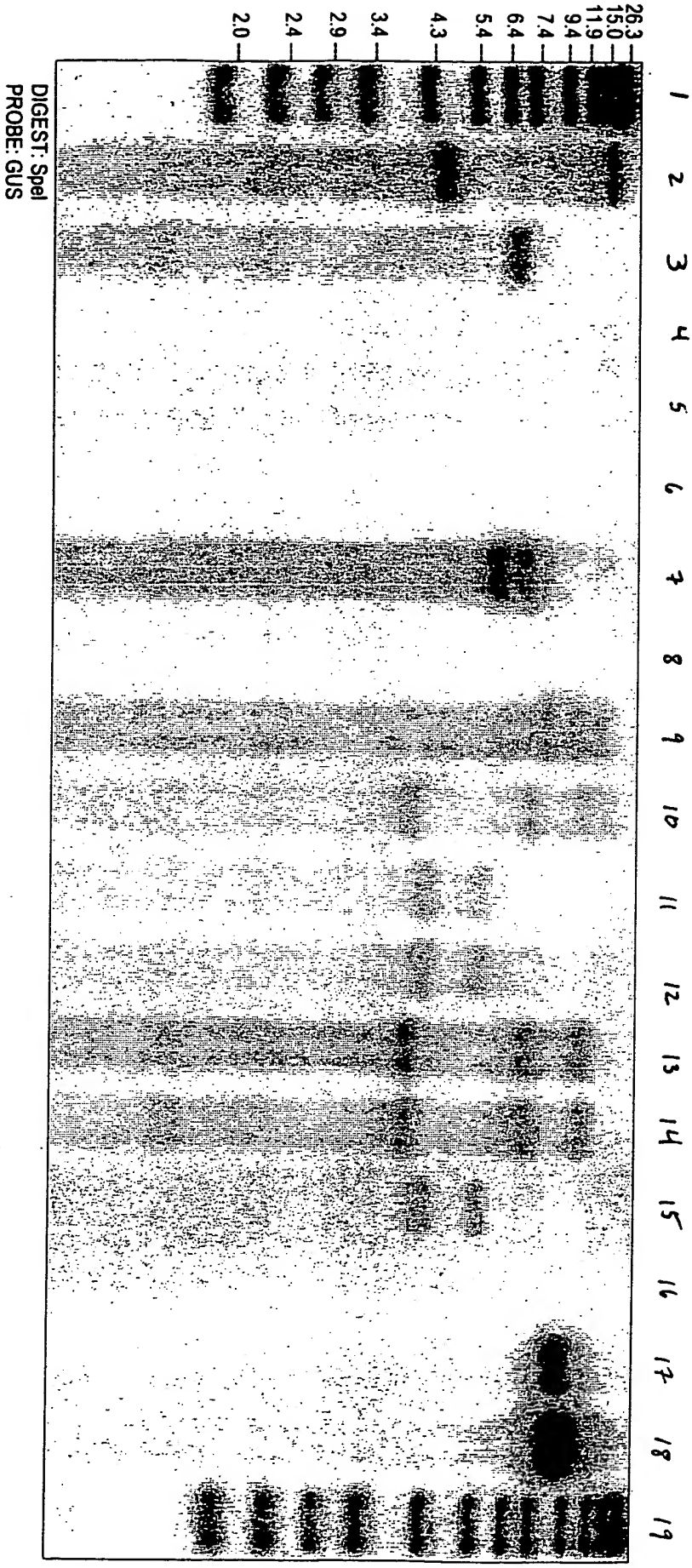
EXHIBIT E

Hi-II



DIGEST: SpeI
PROBE: GUS

EXHIBIT F



PHJ90

EXHIBIT G

PHP38